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SIN1/MIP1 Maintains rictor-mTOR Complex Integrity and Regulates Akt Phosphorylation and Substrate Specificity

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SUMMARY

Mammalian target of rapamycin (mTOR) controls cell growth and proliferation via the raptor-mTOR (TORC1) and rictor-mTOR (TORC2) protein complexes. Recent biochemical studies suggested that TORC2 is the elusive PDK2 for Akt/PKB Ser473 phosphorylation in the hydrophobic motif. Phosphorylation at Ser473, along with Thr308 of its activation loop, is deemed necessary for Akt function, although the regulatory mechanisms and physiological importance of each phosphorylation site remain to be fully understood. Here, we report that SIN1/MIP1 is an essential TORC2/PDK2 subunit. Genetic ablation of *sin1* abolished Akt-Ser473 phosphorylation and disrupted rictor-mTOR interaction but maintained Thr308 phosphorylation. Surprisingly, defective Ser473 phosphorylation affected only a subset of Akt targets in vivo, including FoxO1/3a, while other Akt targets, TSC2 and GSK3, and the TORC1 effectors, S6K and 4E-BP1, were unaffected. Our findings reveal that the SIN1-rictor-mTOR function in Akt-Ser473 phosphorylation is required for TORC2 function in cell survival but is dispensable for TORC1 function.

INTRODUCTION

Cell growth and proliferation are orchestrated by signaling networks in response to environmental cues such as nutrients, growth factors, and hormones. An important player in the control of cell growth is the evolutionarily conserved protein kinase, target of rapamycin (TOR) (Jacinto and Hall, 2003; Sarbassov et al., 2005a; Wullschleger et al., 2006). In addition to being a central regulator of cell growth (size/mass increase), proliferation, apoptosis, and metabolism, mammalian TOR (mTOR) is also linked to the PI3K/PTEN/Akt/TSC signaling pathway, where genetic mutations of many components in this pathway result in the development of a wide variety of cancers. Thus the mTOR pathway is an attractive anticancer drug target (Guertin and Sabatini, 2005; Hay, 2005).

Recent studies have revealed that mTOR, similar to its yeast counterpart, resides in two protein complexes (Inoki and Guan, 2006; Wullschleger et al., 2006). Mammalian TOR complex 1 (TORC1) is rapamycin-sensitive and consists of mTOR, raptor, and mLST8 (GβL), which are the orthologs of yeast TOR1/2, KOG1, and LST8, respectively (Hara et al., 2002; Kim et al., 2002, 2003; Loewith et al., 2002). TORC1 is activated by nutrients, growth factors/hormones, and energy signals and is inhibited by rapamycin. Activation of TORC1 results in phosphorylation of the translational regulators S6K and 4E-BP, which augments protein synthesis (Gingras et al., 2004). Both raptor and mLST8 positively regulate TORC1 functions, but the detailed mechanism of this regulation is unclear. Mammalian TORC2 is rapamycin insensitive and contains mTOR, rictor (mAVO3), and mLST8 (Jacinto et al., 2004; Sarbassov et al., 2004). Yeast TORC2 contains TOR2, AVO3, LST8, and additionally AVO1, AVO2, and BIT61 (Loewith et al., 2002; Reinke et al., 2004; Wedaman et al., 2003). Whether a distantly related gene *hsin1* (*h*uman *S*tress Activated Protein Kinase *In*teracting Protein 1) is a functional equivalent of *AVO1* needs further examination (Loewith et al., 2002; Reinke et al., 2004; Wullschleger et al., 2006). The function of mammalian TORC2 is less defined than that of TORC1 but is believed to be involved in actin cytoskeleton reorganization (Jacinto et al., 2004; Loewith et al., 2002; Sarbassov et al., 2004). Recent biochemical studies showed that TORC2 was able to phosphorylate the growth factor-regulated kinase Akt/PKB (Hresko and Mueckler, 2005; Sarbassov et al., 2005b).

Akt/PKB is a member of the AGC kinase family, which also includes S6K, RSK, SGK, and PKC (Peterson and Schreiber, 1999; Woodgett, 2005). Most members of this

family, including Akt, are phosphorylated at two key residues located at the catalytic site (activation loop or T-loop) and the C-terminal hydrophobic motif (HM) site. Phosphorylation of the HM site promotes docking of the PIF pocket of PDK1 to the HM site and concomitantly leads to the phosphorylation of the T-loop site upon growth factor stimulation and PI3K activation (Biondi, 2004). PDK1 phosphorylates Akt/PKB at Thr308 of its T loop, which is essential for Akt catalytic activity (Alessi et al., 1997; Stephens et al., 1998). As with many members of the AGC kinase family, HM phosphorylation of Akt at Ser473 and T-loop Thr308 phosphorylation has been proposed to be interdependent on each other (Scheid et al., 2002; Toker and Newton, 2000), although there is some opposing evidence (Alessi et al., 1996; Collins et al., 2003; Woodgett, 2005). For example, cells expressing a PDK1 mutant with a defective PIF pocket can still phosphorylate Akt (Biondi et al., 2001; Collins et al., 2003). Moreover, the HM of Akt also displays lower affinity for the PIF pocket of PDK1 in comparison to other PDK1 targets (Frodin et al., 2002). Nevertheless, since dually phosphorylated Akt has higher in vitro kinase activity (Alessi et al., 1996; Scheid et al., 2002), it is presumed that most Akt functions are mediated by the dually phosphorylated Akt. Structural studies of Akt revealed that the HM phosphorylation facilitates interaction of this motif with the N lobe of the catalytic domain of Akt, which in turn promotes a disorder to order transition of the αC helix (Yang et al., 2002a, 2002b). The restructured/ordered αC helix was implied to set the substrate specificity of Akt (Yang et al., 2002a). Despite being widely used as a key indicator of Akt activation, the precise physiological function of Ser473 phosphorylation site remains to be fully understood.

The kinase that phosphorylates the HM site of Akt, often referred to as PDK2, has been controversial. Various kinases have been proposed to act as the PDK2 for Akt, including ILK, DNA-PK, and PKC (Bayascas and Alessi, 2005; Dong and Liu, 2005; Feng et al., 2004; Kawakami et al., 2004; Troussard et al., 2003; Woodgett, 2005). However, despite a lack of genetic evidence, recent biochemical studies have strongly suggested that TORC2 is the elusive PDK2 for Akt Ser473 (Hresko and Mueckler, 2005; Sarbassov et al., 2005b).

Akt plays a central role in controlling cell growth, proliferation, survival, and differentiation by phosphorylating a diverse number of protein substrates that contain a minimal consensus sequence of RXRXXS/T (Scheid and Woodgett, 2001). Among these are proteins implicated in metabolism, cell growth, and proliferation such as GSK3, mTOR, and TSC2, and proteins that play roles in apoptosis such as BAD, ASK1, and the Forkhead family of transcription factors FoxOs (Lawlor and Alessi, 2001). It is therefore important to understand how Akt can control such a diverse array of cellular functions.

Akt is believed to couple the growth-factor-PI3K signaling pathway to the nutrient-regulated TORC1 signaling pathway (Wullschleger et al., 2006). Following growth-factor stimulation, activated Akt suppresses the GTPase-activating protein (GAP) activity of the tumor suppressor TSC2/TSC1 complex by phosphorylating TSC2 (Gao et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002). TSC2 acts as a GAP toward the GTPase Rheb, a positive regulator of the TORC1 signaling branch (Inoki and Guan, 2006; Li et al., 2004; Wullschleger et al., 2006). Thus, Akt acts as an upstream activator of mTOR for TORC1. Since Akt activation may be dependent on TORC2, Akt is also a target of mTOR via TORC2. Hence, the signaling molecules that form a complex with mTOR could specifically link Akt to either TORC1 or TORC2.

In this study, we identified SIN1/MIP1 as a key TORC2 component and regulator of the Akt pathway that positively controls Akt-Ser473 phosphorylation and activation. These studies highlight the role of the SIN1-rictor-mTOR complex in defining the function and specificity of Akt.

RESULTS

Human SIN1 Is a Component of TORC2 but Not TORC1

To understand the molecular mechanism of TORC1 and TORC2 regulation, we immunoprecipitated either rictor or raptor and identified coimmunoprecipitated proteins by mass spectrometry. Both rictor and raptor coimmunoprecipitated mTOR (Figure 1A). A 70 kDa protein band that specifically associated with rictor but not raptor was identified as SIN1 (SAPK interacting protein 1, also previously identified as MIP1 for MEKK2 interacting protein 1), a human ortholog of *Schizosaccharomyces pombe* spSIN1 (Cheng et al., 2005b; Schroder et al., 2005; Wilkinson et al., 1999). The weaker 70 kDa band seen in the raptor immunoprecipitates was HSP70, and no trace of SIN1 peptide was identified. Reciprocally, we performed proteomic analysis of the SIN1 complex and detected rictor and mTOR but not raptor (Figures 1A and 1B). Further biochemical analysis indicated that SIN1 may participate in TORC2 but not TORC1 function (Figure S1).

Targeted Inactivation of *sin1* Gene by Homologous Recombination

There is so far no genetic evidence in mammals demonstrating the role of mTOR partners in TORC signaling. To understand more thoroughly the in vivo function of SIN1, we generated mice with targeted deletion of *sin1* by homologous recombination (Figure 2A). The targeting event was verified by Southern blotting, PCR-based genotyping, and Western blotting analysis (Figures 2B–2D). Although $SIN^{+/-}$ heterozygous mice breed and develop normally with no gross differences from wild-type mice, the *sin1*-deleted mice were embryonic lethal due to early developmental problems (these findings will be described elsewhere).

SIN1 Is Required for Ser473 but Not for Thr308 Phosphorylation of Akt

Due to embryonic lethality, we established $SIN1^{-/-}$ murine embryonic fibroblasts (MEFs) from E10 embryos (Figure 2D) to investigate the role of SIN1 in mTOR function.

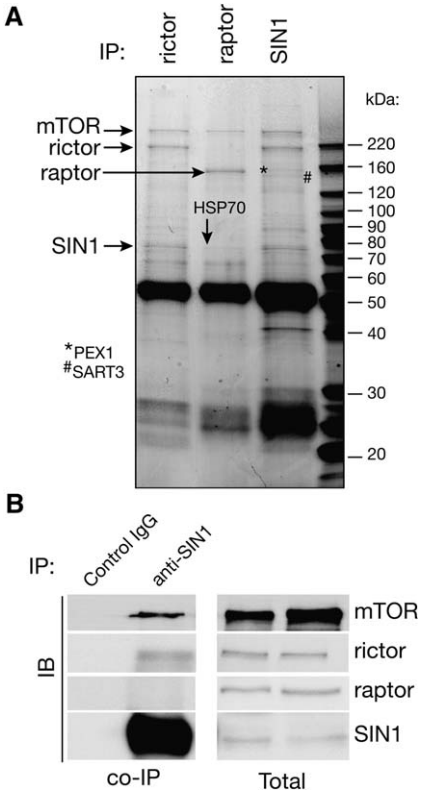

Figure 1. SIN1 Is a Subunit of TORC2

(A) Proteomic analysis of endogenous TORC2. Extracts from one billion HeLa cells were immunoprecipitated with rictor-, raptor-, or SIN1-specific antibodies. Coimmunoprecipitated proteins were analyzed and identified by mass spectrometry. SIN1, rictor, raptor, and mTOR are indicated. PEX1 and SART3 coimmunoprecipitated with SIN1 and migrated at a similar position as raptor, while HSP70 migrated at the same position as SIN1.

(B) SIN1 interacts with rictor and mTOR, but not raptor, in vivo. HEK293T cell extracts were subjected to immunoprecipitation by an anti-SIN1 antibody or a control IgG antibody. Coimmunoprecipitated (coIP) or total proteins (total) were immunoblotted with specific antibodies to each of the TORC components.

In wild-type control and starved cells (all the cells were serum starved for 12 hr, followed by 60 min incubation with PBS for nutrient starvation) that were restimulated with serum or insulin, Akt became phosphorylated at Ser473 (Figure 3A). In SIN1$^{-/-}$ cells, however, the phosphorylation of Akt at Ser473 was undetectable even after prolonged serum stimulation (Figure 3A and data not shown). Since SIN1 is not a kinase and has no known enzymatic activity, these results strongly suggest that SIN1 is the key regulator of PDK2 that phosphorylates Akt at Ser473.

We next examined the Akt T-loop Thr308 phosphorylation in wild-type and SIN1$^{-/-}$ cells. We found that although Ser473 phosphorylation was completely abolished in the SIN1$^{-/-}$ cells, Thr308 phosphorylation of Akt was not blocked (Figure 3A). These results indicate that SIN1 is not essential for Akt Thr308 phosphorylation and that the Ser473 phosphorylation is not a prerequisite for Thr308 phosphorylation.

Akt, Singly Phosphorylated at Thr308, Remains Active and Functional in Response to Growth Factor Stimulation in SIN1$^{-/-}$ Cells

Since Akt Thr308 phosphorylation was not dependent on Ser473 phosphorylation in SIN1$^{-/-}$ cells, we then asked whether this Thr308 singly phosphorylated Akt is still active and whether lack of Ser473 phosphorylation would have a global effect on the phosphorylation of Akt targets. Using an in vitro kinase assay, we found that Akt retained a substantial amount of enzymatic activity in SIN1$^{-/-}$ cells, although lower than that in wild-type cells (Figure 3B). Thus, singly phosphorylated (Thr308) Akt is an active but weaker enzyme.

To investigate whether this partially active Akt is functional in vivo, we first examined the in vivo phosphorylation of GSK3. Surprisingly, phosphorylation of GSK3α at Ser21 and GSK3β at Ser9 in response to serum or insulin was only marginally affected in the SIN1$^{-/-}$ cells (Figure 3C). We also did not observe a difference in the kinetics of GSK3 phosphorylation between the wild-type and SIN1$^{-/-}$ cells (data not shown). These results suggest that the singly Thr308-phosphorylated form of Akt may be partially functional in vivo.

We also examined phosphorylation of TSC2, another Akt target (Manning et al., 2002), at the Akt target sites Ser939 and Thr1462 and found no significant difference between wild-type and SIN1$^{-/-}$ cells upon serum and insulin stimulation (Figure 3C). Furthermore, we compared the proliferation rates of several sets of MEFs with wild-type, SIN1$^{+/-}$, and SIN1$^{-/-}$ genotypes established from E10 embryos. No significant difference in the cell-doubling time of these MEFs was observed (Figure 3D). Finally, we did not find any size difference of wild-type and SIN1$^{-/-}$ cells grown under starvation or normal growth conditions (Figure 3E and data not shown). Hence, the growth factor-induced GSK3 and TSC2 phosphorylation may not fully depend on Akt-Ser473 phosphorylation.

Phosphorylation at Thr24/Thr32 of FoxO1/FoxO3a, an Akt Target for the Cell-Survival Pathway, Is Defective in SIN1$^{-/-}$ Cells

To identify a function that could be linked specifically to Akt-Ser473 phosphorylation, we further examined known Akt substrates that may have defective phosphorylation in SIN1$^{-/-}$ cells. We found that phosphorylation of FoxO1/3a (also called FKHR/FKHRL1) (Greer and Brunet, 2005), was affected in SIN1$^{-/-}$ cells. In particular, phosphorylation of FoxO1/3a at Thr24/Thr32 was significantly decreased in the absence of SIN1 under normal growing and restimulated conditions (Figure 4A). However, we did not observe complete inhibition of phosphorylation at this site, indicating that it could be partially induced by the singly Thr308-phosphorylated Akt, or compensated by another AGC family-related kinase such as SGK1. We also found that

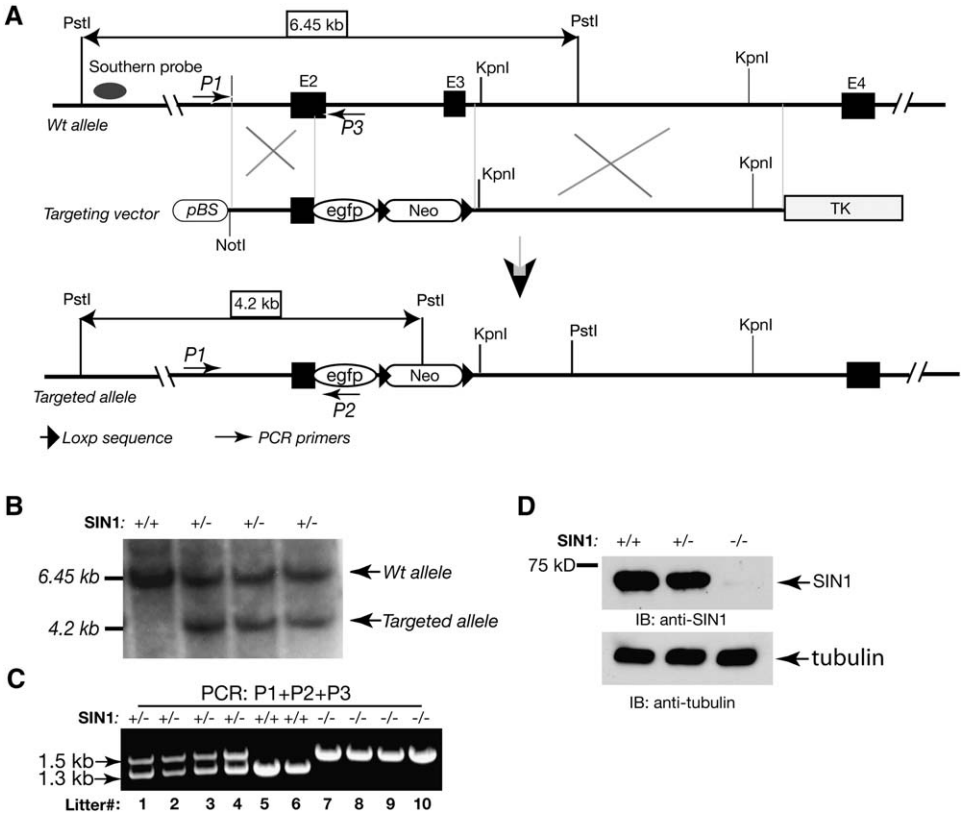

Figure 2. Targeted Mutation of the Murine *sin1* Gene by Homologous Recombination

(A) Diagram of the *sin1* locus and the targeting vector. The egfp-Neo cassette flanked by the two targeting arms and the TK cassette is illustrated. The filled squares are *sin1* exons. An oval indicates the *sin1* DNA region used as a probe for Southern blot analysis. The locations of PCR primers are indicated.

(B) Southern blot analysis of the targeted allele. The 6.45 kb Pst1-Pst1 wild-type allele and the 4.2 kb targeted allele are indicated.

(C) PCR genotyping of an E10 litter. The 1.3 kb (wild-type) and 1.5 kb fragments (targeted allele) were amplified using primers P1-P3 and P1-P2, respectively.

(D) Analysis of murine embryonic fibroblasts (MEFs) by immunoblotting. Total cell lysates from serum-starved wild-type(+/+), heterozygous (+/−), and homozygous mutant (−/−) MEFs were analyzed by immunoblotting using a SIN1 specific antibody K87 and tubulin as loading control.

the recombinant FoxO1 could not be phosphorylated at Thr24/32 in vitro by an Akt mutant harboring an alanine mutation in either Thr308 or Ser473, whereas a Ser473 to Ala mutated Akt could still phosphorylate recombinant GSK3 (Figure S2). These results indicate that Akt-Ser473 phosphorylation is required for FoxO1/3a phosphorylation at Thr24/32. Interestingly, FoxO1/3a Ser256 phosphorylation was normal in SIN1$^{-/-}$ cells (data not shown).

To search for other potential Akt targets that may be defective in SIN1$^{-/-}$ cells, we used a pan-Akt substrate phosphoantibody. Phosphorylation of most of the Akt substrates detected by this phosphoantibody was similarly induced and therefore is SIN1 independent (Figure 4B). However, the phosphorylation of at least two proteins with an apparent molecular weight of 46 and 48 kDa was severely defective in the SIN1$^{-/-}$ cells. The identity of these proteins is unknown, but they do not appear to be GSK3 α/β. Probing the same membrane with an anti-p-GSK3α/β antibody revealed that although GSK3 comigrated with the p46 band, there was no defect in its phosphorylation in the SIN1$^{-/-}$ cells (data not shown; see also Figure 3C). Together these results demonstrate that lack of hydrophobic motif (HM)-site phosphorylation does not have a global effect on Akt substrate phosphorylation but affects only a subset of Akt targets in vivo. To our knowledge, this is the first time that specific functions of Akt Thr308 and Ser473 phosphorylation have been clearly uncoupled.

SIN1 Deficiency Leads to Increased Susceptibility of Cells to Stress-Induced Apoptosis

Akt has been well characterized as a prosurvival molecule, and part of this function is mediated through its suppression of the activity of the FoxO proteins (Greer and Brunet, 2005). To investigate whether SIN1 deficiency confers increased sensitivity to stress-induced apoptosis, we induced cellular stress in the SIN1$^{-/-}$ MEFs by using hydrogen peroxide (H_2O_2) and the DNA damage-inducing agent,

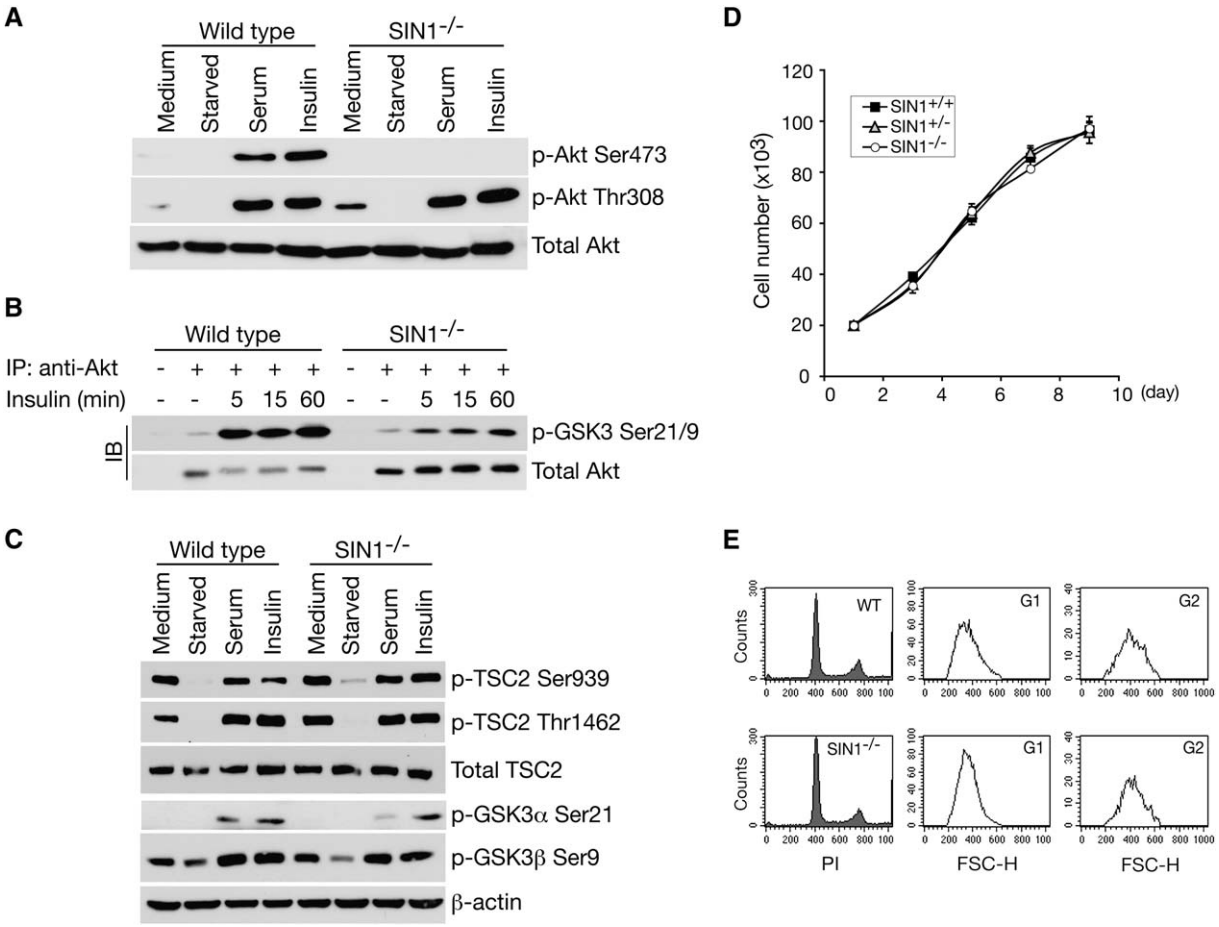

Figure 3. SIN1 Is Required for Ser473 but Not for Thr308 Phosphorylation of Akt but It Is Dispensable for GSK3 and TSC2 Phosphorylation and Cell-Growth/Proliferation Control

(A) SIN1 is required for Akt Ser473 but not Thr308 phosphorylation. Wild-type and SIN1$^{-/-}$ MEF lysates from normal growing (medium), starved, or starved then restimulated conditions were analyzed for p-Akt Ser473, p-Akt Thr309, or total Akt by immunoblotting.

(B) SIN1 deletion decreases Akt enzymatic activity in vitro. Total Akt from starved (−), or starved then insulin restimulated cells (5, 15, 60 min restimulation) was immunoprecipitated for in vitro kinase assay. Phosphorylation of recombinant GSK3 at pSer21/9 and total immunoprecipitated Akt was determined by immunoblotting.

(C) SIN1 is not required for phosphorylation of the Akt target sites on TSC2 and GSK3. Wild-type and SIN1$^{-/-}$ MEFs were treated as described in (A) and analyzed for TSC2 (p-Ser939 and p-Thr1462), GSK3α/β (p-Ser9/21) phosphorylation, and total TSC2 or β-actin expression, by immunoblotting.

(D) SIN1$^{-/-}$ cells grow similarly as wild-type cells. Wild-type, SIN1$^{+/-}$ and SIN1$^{-/-}$ MEFs grown in complete medium were counted on the indicated days. Data are means ± standard deviation of triplicates plates.

(E) Wild-type and SIN1$^{-/-}$ cells display similar cell size. Wild-type and SIN1$^{-/-}$ MEFs were stained by propidium iodide (PI) and subjected to FACS analysis. DNA content (PI) and forward scatter height (FSC-H) distribution of MEFs during the G1 and G2 phase of the cell cycle are shown.

etoposide, which are known to induce cell death in an Akt-FoxO pathway-dependent manner (Brognard et al., 2001; Brunet et al., 1999, 2004). Upon H_2O_2 treatment, whereas 70%–100% of wild-type cells remained viable, only 20%–50% of SIN1 knockout cells survived (Figure 4C). In etoposide-treated cells, there was a 2-fold increase in the number of cells that underwent apoptosis after 48 hr in SIN1 knockouts (Figure 4D). Thus, we find that the SIN1$^{-/-}$ cells were more sensitive to stress-induced apoptosis, suggesting that Akt-Ser473 phosphorylation plays an important role in cell survival (Figures 4C and 4D).

SIN1 Is Not Required for the raptor-mTOR Function

Although SIN1 does not bind to TORC1, it is still possible that SIN1 may regulate TORC1 indirectly. We therefore examined the phosphorylation of TORC1 targets in the SIN1$^{-/-}$ cells. The phosphorylation of S6K at Thr389, which is the TORC1-mediated site, was not inhibited in the SIN1$^{-/-}$ cells (Figure 5A). In fact, we observed a slight increase in Thr389 phosphorylation in response to serum or insulin stimulation in the absence of SIN1. Likewise, phosphorylation of another translational regulator, 4E-BP1, at the mTOR target site Thr37/46 (Gingras et al.,

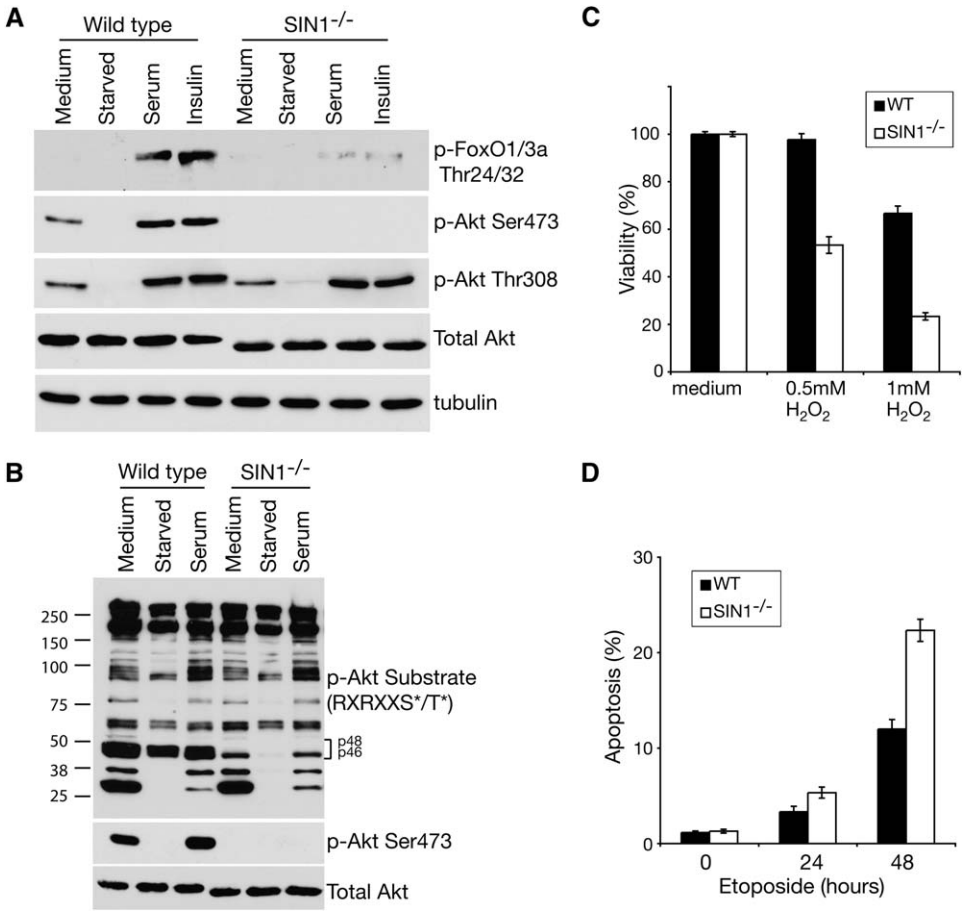

Figure 4. SIN1-Deficient Cells Have Defective FoxO1/3a Thr24/Thr32 Phosphorylation and Are More Susceptible to Stress-Induced Apoptosis

(A) SIN1 is essential for FoxO1/3a Thr24/Thr32 phosphorylation. Wild-type and SIN1$^{-/-}$ MEFs were treated as described in Figure 3A. Phosphorylation of FoxO1/3a, p-Akt Ser473, p-Akt Thr308, total Akt, and tubulin were determined by immunoblotting.

(B) Phosphorylation of putative Akt substrates in SIN1$^{-/-}$ cells. Wild-type and SIN1$^{-/-}$ MEFs were treated as described in A. Total Akt, p-Akt Ser473, and the phosphorylation of putative Akt substrates were determined by immunoblotting.

(C and D) SIN1$^{-/-}$ cells have increased susceptibility to stress-induced apoptosis. In (C), wild-type and SIN1$^{-/-}$ MEFs were treated with 0.5 mM or 1mM H_2O_2, and cell viability was determined 10 hr later. In (D), wild-type and SIN1$^{-/-}$ MEFs were treated with 20 μM etoposide for the indicated times, and the percentage of apoptotic cells were determined by FACS analysis. Error bars represent standard deviation.

1999), was also not impaired in SIN1$^{-/-}$ cells (Figure 5A). Hence, SIN1 appears to be essential for TORC2 function but is dispensable for TORC1 function.

Since SIN1 is not required for TORC1 function, we next examined whether the SIN1-mediated Akt-Ser473 phosphorylation is responsive to growth-factor stimulation in the absence of nutrients such as amino acids and glucose. Akt Ser473 was strongly induced in wild-type cells by different growth factors such as the platelet-derived growth factor, epidermal growth factor, and insulin, even in the absence of amino acids and glucose (Figure 5B). In SIN1$^{-/-}$ cells, Akt-Ser473 phosphorylation was not induced by any type of stimulus. In contrast, Thr308 phosphorylation was induced by growth factors in both wild-type and SIN1$^{-/-}$ cells, indicating that the SIN1 deletion specifically affects Akt-Ser473 phosphorylation in response to growth factors. These results also indicate that SIN1 may mediate growth-factor signaling for Akt-Ser473 phosphorylation independent of nutrient signals.

SIN1 Is Essential for rictor-mTOR Interaction and Interacts with Akt

Next we examined if SIN1 could regulate the PDK2 function by controlling the rictor and mTOR interaction. Whereas rictor interacted with mTOR in wild-type cells, this interaction was impaired in SIN1$^{-/-}$ cells under starved or restimulated conditions (Figure 5C). In contrast, the raptor-mTOR interaction was not affected in the absence of SIN1 (Figure 5D). Taken together, these results indicate that SIN1 is essential for TORC2 integrity.

To investigate if SIN1 could serve as a scaffold to present Akt to TORC2 to mediate Akt phosphorylation at

Ser473, we examined SIN1 and Akt interaction. As shown in Figures 5E and 5F, SIN1 was able to associate with Akt under normal growth, starvation, or restimulated conditions. Although SIN1 and Akt interacted under starved condition, the SIN1 bound Akt was not phosphorylated on Ser473 (Figure 5E). In contrast, we did not detect Akt and rictor interaction by either coimmunoprecipitation (coIP) (Figure S3) or mass spectrometry analysis (data not shown), suggesting that Akt does not form a stable complex with rictor or TORC2. These results suggest that SIN1 may serve as a scaffold for Akt in addition to being a regulator of TORC2.

Expression of SIN1 Rescues the Defects in SIN1 Mutant Cells

To confirm that the defects we observed in the SIN1$^{-/-}$ MEFs were indeed due to the SIN1 deletion, we reconstituted the SIN1$^{-/-}$ cells with full-length *sin1* cDNA and analyzed the TORC2-Akt function (Figure 6A). Expression of the full-length HA-tagged SIN1, but not the empty vector, was able to restore Akt-Ser473 phosphorylation (Figure 6B). The interaction of rictor and mTOR was also restored (Figure 6C). Finally, the phosphorylation of FoxO1/3a at Thr24/Thr32 and the putative p46/48 protein in the reconstituted cells was likewise restored (Figures 6D and 6E). Together these results confirm that SIN1 is an essential TORC2 subunit and that it is critical for Akt activation and function.

DISCUSSION

Our studies have identified SIN1 as a crucial subunit of the TORC2 protein complex and that the SIN1-rictor-mTOR complex is likely the principal if not the sole phosphoinositide-dependent protein kinase 2 (PDK2) for Akt. We provide here the first mammalian genetic evidence for distinct functions of the two mTOR complexes and show that phosphorylation of Akt at the HM site by TORC2 serves a specific cellular function.

A key finding in our study is that in the absence of SIN1, phosphorylation of Ser473 but not Thr308 of Akt is abolished, demonstrating that the PDK1-mediated Akt Thr308 phosphorylation is not dependent on prior Ser473 phosphorylation by PDK2. In contrast to our data, another study (Sarbassov et al., 2005b) found that both Ser473 and Thr308 phosphorylation of Akt was diminished in rictor and mTOR knockdown cells. The reason for the discrepancy with our findings is unclear, but we do not exclude the possibility that uncoupled rictor and mTOR could be required for PDK1 regulation. Since we found that in the absence of Ser473 phosphorylation, Thr308 phosphorylation occurs more readily in SIN1$^{-/-}$ cells than that in wild-type cells (data not shown), the HM site phosphorylation of Akt may still influence how the T-loop Thr308 is phosphorylated. This result supports the view that these two phosphorylation sites may directly or indirectly influence the status of the phosphorylation of the other (Bayascas and Alessi, 2005; Scheid et al., 2002; Woodgett, 2005; Yang et al., 2002a, 2002b).

The dual phosphorylation of Akt at the HM and T-loop sites that synergistically activates Akt in vitro is not necessarily required for all Akt functions. Specifically, we found that phosphorylation of FoxO1/3a at Thr24 and at least two unidentified p46 and p48 proteins was defective in SIN1$^{-/-}$ cells, while singly Thr308-phosphorylated Akt was sufficient to phosphorylate other Akt targets. As the phosphorylation of FoxO1/3a at multiple sites including Thr24 has been proposed to promote the nuclear exclusion and binding to 14-3-3 proteins of FoxOs (Barthel et al., 2005; Greer and Brunet, 2005), a fully active, doubly phosphorylated Akt may enhance or sustain inhibition of these transcription factors to promote cell survival upon growth-factor stimulation. In agreement with this hypothesis, we found increased susceptibility of SIN1$^{-/-}$ cells to stress-induced apoptosis. Whether the unidentified p46 and p48 proteins are also involved in cell survival remains to be elucidated.

Thus, differently activated forms of Akt may regulate the numerous Akt targets in a specific manner. Akt binding proteins and/or the localization of Akt to a specific cellular compartment could regulate its activity and confer specificity (Brazil et al., 2002). It is however also possible that other AGC-type kinases such as PKC and or RSK, which have been proposed to phosphorylate GSK3 at the same inhibitory sites (Jope and Johnson, 2004), could compensate for the function of doubly phosphorylated Akt. In this regard, it would be interesting to determine if SIN1 may also regulate PKC and RSK. Our finding that singly phosphorylated Akt at Thr308 retains most of Akt functions also provides a cautionary note for the use of Akt phosphorylation at Ser473 as a sole indicator for Akt activation and function.

Our studies also revealed that SIN1 is not required for TORC1 activation. In fact, we observed a slightly enhanced S6K1 phosphorylation at Thr389 in both SIN1 knockout (Figure 5A) and knockdown (data not shown) cells. It was recently shown that in the absence of TSC2, Akt-Ser473 phosphorylation is also impaired. This defect was attributed to a negative feedback regulation of the insulin receptor substrates, IRS, by S6K, whose phosphorylation was augmented in TSC2-deficient cells due to the enhanced TORC1 activity (Harrington et al., 2004; Roux et al., 2004; Shah et al., 2004). However, the defective Akt-Ser473 phosphorylation in SIN1$^{-/-}$ cells is unlikely due to this negative feedback loop since PDK1 signaling remained unaffected (e.g., Akt phosphorylation by PDK1 at Thr308). Thus, increased S6K activity may not necessarily impair insulin signaling but could occur only when S6K is persistently activated, as would occur in the absence of negative regulation by TSC2. Alternatively, the increase in S6K activity in the SIN1$^{-/-}$ cells may not be sufficient to attenuate insulin signals.

Another indication that SIN1 is not required for TORC1 activation was the observation that TSC2 is also phosphorylated normally in SIN1$^{-/-}$ cells. Akt has been

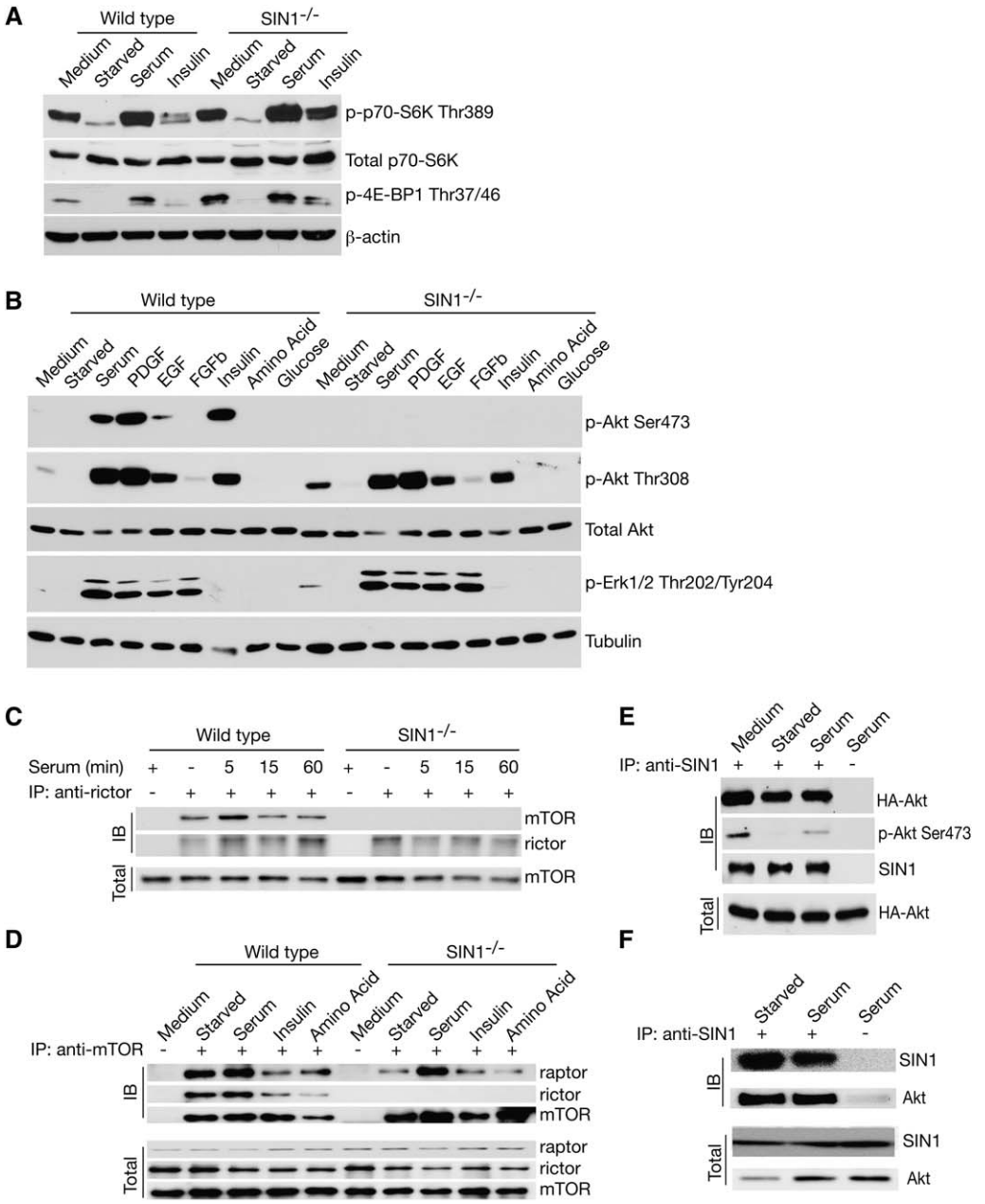

Figure 5. SIN1 Is Not Required for Raptor-mTOR Function but Is Required for Multiple Growth Factor-Mediated Akt-Ser473 Phosphorylation and Rictor-mTOR Assembly

(A) SIN1 is not required for phosphorylation of S6K and 4E-BP1. Wild-type or SIN1$^{-/-}$ MEFs were grown in complete medium, or starved, or starved then restimulated with serum or insulin for 15 min. Total cell lysates were analyzed for p-S6K Thr389, p-4E-BP1 Thr37/46, total S6K, or β-actin by immunoblotting.

(B) SIN1 is required for multiple growth factor-mediated Akt-Ser473 phosphorylation independent of nutrient input. Wild-type or SIN1$^{-/-}$ MEFs were starved of serum and by nutrient (in PBS for the last 90 min) and then restimulated as indicated. p-Akt Ser473, p-Akt Thr308, total Akt, p-ERK1/2, and tubulin were determined by immunoblotting.

(C) The rictor-mTOR interaction is disrupted in SIN1 deficient cells. Wild-type and SIN1$^{-/-}$ MEFs were either starved or restimulated with serum for various time points as indicated. Rictor was immunoprecipitated and the associated mTOR and total mTOR from the cell lysates were determined by immunoblotting.

(D) The raptor-mTOR interaction is not altered in SIN1-deficient cells. Wild-type and SIN1$^{-/-}$ MEFs were starved and then restimulated for 15 min as indicated. mTOR was immunoprecipitated, and associated rictor or raptor and total protein levels of raptor, rictor, and mTOR were analyzed by immunoblotting.

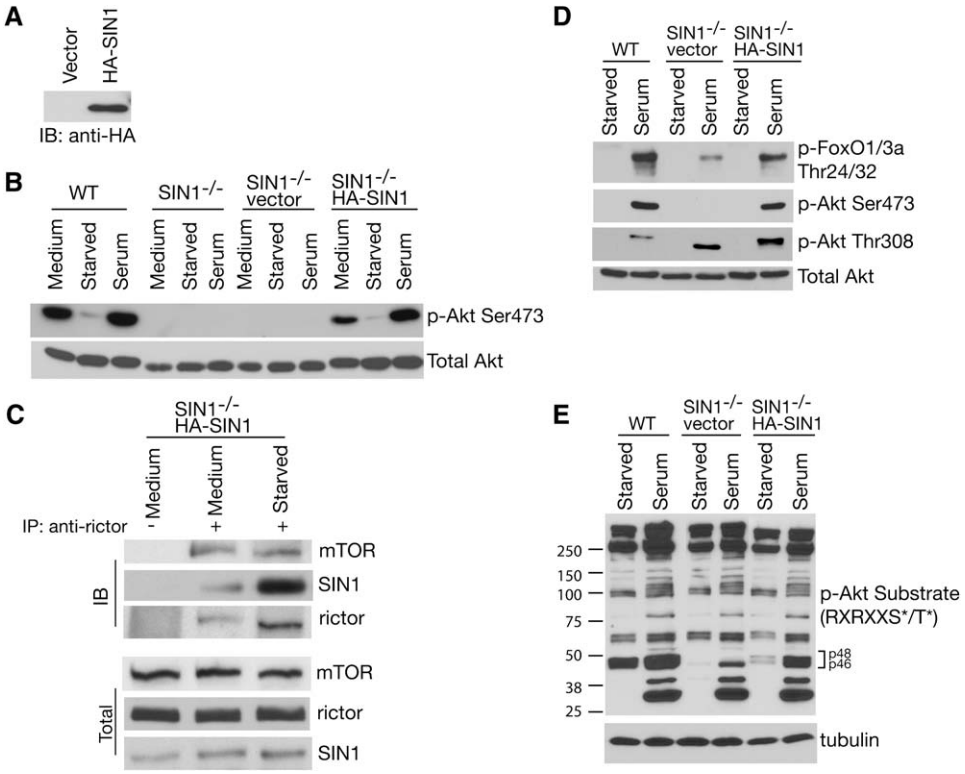

Figure 6. Restoration of SIN1$^{-/-}$ MEFs with SIN1 Expression Vector

(A) Expression of HA-SIN1 in SIN1$^{-/-}$ MEFs. SIN1$^{-/-}$ MEFs were infected with either an empty vector, or HA-tagged SIN1 expression vector. HA-SIN1 expression was analyzed by immunoblotting.

(B) Expression of HA-SIN1 in SIN1$^{-/-}$ MEFs restores Akt-Ser473 phosphorylation in the presence of serum. Wild-type, SIN1$^{-/-}$ MEFs, or HA-SIN1-reconstituted SIN1$^{-/-}$ MEFs, as described in (A), were grown either in complete medium, or starved, or starved then restimulated with serum for 15 min and then analyzed for Akt-Ser473 phosphorylation and total Akt by immunoblotting.

(C) Expression of HA-SIN1 in SIN1$^{-/-}$ MEFs restores rictor-mTOR interaction. Total rictor was immunoprecipitated from the HA-SIN1-expressing SIN1$^{-/-}$ MEFs, grown either in complete medium or starved cells. The coimmunoprecipitated mTOR and SIN1 was analyzed by immunoblotting.

(D and E) Expression of HA-SIN1 in SIN1$^{-/-}$ MEFs restores phosphorylation of FoxO1/3a and p46/p48 proteins. Wild-type, SIN1$^{-/-}$ MEFs or HA-SIN1-reconstituted SIN1$^{-/-}$ MEFs were starved or starved then restimulated with serum for 15 min and then analyzed for p-FoxO1/3a T24/T32 phosphorylation (D) or pan p-Akt substrate phosphorylation (E). p-Akt Ser473, p-Akt Thr308, total Akt, and tubulin were determined by immunoblotting.

proposed to be a positive regulator of TORC1 by inactivating TCS2. However, Akt-Ser473 phosphorylation is not required for TORC1 activation, suggesting that either the singly Thr308-phosphorylated Akt fulfills this function or Akt may play a minor role in TSC2 phosphorylation and inactivation. The contribution of Akt on TORC1 activation through TSC2 is still unclear since other pathways besides Akt are also implicated in TSC2 regulation. TSC2 is also phosphorylated and inactivated by other kinases such as AMPK, ERK1/2, and RSK at sites other than the Akt target residues to positively regulate TORC1 signaling (Inoki et al., 2003; Ma et al., 2005; Roux and Blenis, 2004). Other studies also suggest that Akt activation is not necessary for S6K activity (Dufner et al., 1999; Radimerski et al., 2002) and that the Akt-phosphorylation sites on TSC2 do not appear to critically affect normal *Drosophila* development (Dong and Pan, 2004).

As TSC2 remains phosphorylated (and presumably inactive) in the absence of Akt-Ser473 phosphorylation in SIN1 knockout cells, what accounts for the slightly enhanced S6K phosphorylation? It is conceivable that when TORC2 integrity is compromised, TORC1 is upregulated due to a more stable mTOR-raptor binding (Hay, 2005; Sarbassov et al., 2005b). This would suggest that mTOR could be a limiting factor in the formation of the complexes and that the balance of the two complexes is

(E) SIN1 interacts with Akt. HEK293T cells were transfected with HA-tagged Akt. Forty-eight hour posttransfection, cells were starved or starved then restimulated. HA-Akt that was coimmunoprecipitated with SIN1, p-Akt Ser473, and total AH-Akt was determined by immunoblotting.

(F) SIN1 interacts with endogenous Akt. Starved or starved then restimulated HEK293T cells were lysed and immunoprecipitated with an anti-SIN1 antibody. The immunocomplex and total lysates were analyzed by immunoblotting.

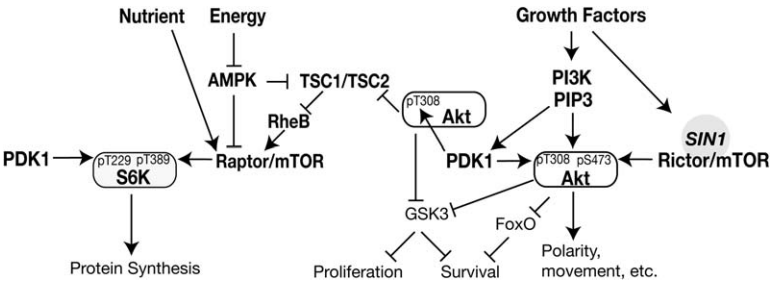

Figure 7. A Model Illustrating the Function of SIN1 in Regulating the Rictor-mTOR Integrity and Akt Specificity

critical for normal responses to growth cues. Although it remains to be shown, an interesting alternative explanation is that SIN1 could negatively regulate TORC1.

SIN1 was originally identified as a human protein that modulates yeast RAS function (Colicelli et al., 1991). In *Dictyostelium*, RIP3 (AVO1/SIN1 ortholog) binds to activated Ras through its Ras binding domain (RBD), which is also required for chemotaxis (Lee et al., 2005, 1999). In both mammals and yeast, SIN1/MIP1 binds serine/threonine kinases MEKK2/3 and SAPK/JNK in the MAPK modules (Cheng et al., 2005b; Schroder et al., 2005; Wilkinson et al., 1999). SIN1 inhibits MEKK2/JNK signaling by forming a complex with the inactive and nonphosphorylated MEKK2, thereby preventing its dimerization and activation (Cheng et al., 2005a, 2005b). It is not clear if the SIN1-mediated TORC2 function involves the MEKK/MAPK cascades, although there is appealing evidence suggesting that the mTOR-Akt pathway is regulated by MAPKs (Ma et al., 2005). However, Akt Thr308 and Ser473 phosphorylation is not affected in either MEKK2-deficient or -activated cells (data not shown). In addition, MEKK2 did not interact with either mTOR or rictor (data not shown). Nevertheless, consistent with our previous finding (Cheng et al., 2005b), we found enhanced JNK and p38 activation in SIN1$^{-/-}$ cells, suggesting a parallel function of SIN1 in the MAPK pathway (data not shown).

Akt and S6K are members of the AGC family of protein kinases. A common theme of their activation is through dual phosphorylation of the T-loop site by PDK1 and the HM site by TORC1 (for S6K) and TORC2 (for Akt). We therefore propose that these two AGC kinases are regulated upon nutrient- and growth-factor stimulation by PDK1 and the TORCs in a parallel fashion as depicted in Figure 7. Phosphorylation of other HM site-regulated AGC kinases such as PKC, RSK, and SGK may be a common feature in mTOR signaling, and whether other members of this family could serve as mTOR substrates deserves further investigation. In this regard, since the pan-Akt substrate antibody is likely to cross react with phosphorylated substrates of AGC kinases besides Akt, it is possible that the phosphorylation defect of FoxO or p46/p48 in SIN1$^{-/-}$ cells may be a combined impairment of Akt and other AGC kinases such as PKC or SGK.

Finally, as our study suggests that the HM and T-loop phosphorylation of Akt may serve distinct functions, a closer re-examination of the role of these two Akt-phosphorylation sites is likely to shift in many ways some views on the role of Akt in various pathological processes such as cancer and diabetes. SIN1 is an important regulator of Akt and perhaps many other pathways as well. SIN1 is essential for early embryonic development; hence, SIN1's function in vivo is likely complex. Future investigations to define the specific role of SIN1 in Akt regulation and in various biological settings should yield important insights into the regulation of growth and development at the organism level.

EXPERIMENTAL PROCEDURES

Proteomic Analysis of TORC2

Antibody isolation and the mass spectrometric identification of associated proteins of rictor, raptor, SIN1, and mTOR complexes were done as described before (Jung et al., 2005) with a minor modification, in which 0.3% of CHAPS detergent was substituted for NP-40 and Triton X-100 in the binding and washing buffer.

Targeted Disruption of sin1 Gene by Homologous Recombination

The *sin1* targeting arms were isolated from a BAC clone identified by screening a Research Genetics (Invitrogen, Carlsbad, CA) BAC library using *sin1* cDNA as a probe. The targeting vector was constructed by replacing part of the exon 2 and exon 3 of the *sin1* gene with an egfp-PGKNeo cassette using standard molecular procedures. The linearized targeting construct was electroporated into AB1 ES cells, and the targeted clones were selected with G418 and gancyclovir. Resistant clones were screened for homologous recombination by PCR and confirmed by Southern blot analysis. For the generation of null mice, two independent SIN1$^{+/-}$ embryonic stem (ES)-cell clones were injected into C57BL/6 blastocysts. Chimeras were further bred with C57BL/6 female mice for germline transmission. SIN1 heterozygous mice were backcrossed more than five generations to the C57BL/6 background in this study.

Cell Culture, Stimulation, Transfection, and Lysis

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and transfected as described (Jacinto et al., 2004). MEFs were established from embryos at embryonic day 10 and maintained in DMEM with 15% FBS as described (Yang et al., 2001). MEFs used for all experiments were primary cells between passages 3 and 7. Cells were serum starved for 12 hr, followed by 60 min incubation with phosphate buffered saline for nutrient starvation unless otherwise indicated, before being restimulated with one or more of the following: 5.2 μg/mL leucine, 3× mixture (relative to concentration in DMEM) of essential and nonessential amino acids, 1 μg/mL insulin, 10% serum, 20 ng/mL PDGF, 100 ng/mL EGF, 10 ng/mL FGFb, or 50 mM glucose. MEFs were infected with retrovirus expressing HA-SIN1 as described (Zhang et al., 2006). Stable cell lines were established by fluorescence-activated cell sorting (FACS). Except for raptor immunoprecipitation,

in which cells were lysed in buffer containing 0.3% CHAPS detergent, cells were lysed for coIP as described (Jacinto et al., 2004).

Immunoprecipitation, Immunoblotting, and Kinase Assay

Total cell extracts harvested from a 10 cm plate were used for each immunoprecipitation condition. Crude extracts, typically in a 500 μl volume, were cleared by 10 min centrifugation at 800g, and precleared with Protein A or G Sepharose (GE Healthcare, Uppsala, Sweden) for 30 min. Precleared lysates were subjected to immunoprecipitation by incubating with the corresponding primary antibody (mTOR N5D11 [IBL, Japan], myc [Upstate, Charlottesville, VA], rictor 15T, raptor 36T (Jacinto et al., 2004), rictor BL2178 (Bethyl Laboratory, Montgomery, TX), SIN1 K87 (Cheng et al., 2005b) or control IgG (for mock IPs), and followed by immunoblotting. To assay enzymatic activity in vitro, an Akt kinase Assay Kit and recombinant GSK3 (Cell Signaling, Danvers, MA) or FoxO (Upstate, Lake Placid, NY) were used. All phosphoantibodies were obtained from Cell Signaling.

Proliferation, Cell Size, and Cell Viability Assay

For cell proliferation, MEFs were seeded at 20,000/well on a 12-well plate and harvested every other day. Cell proliferation rates were measured by direct cell counting. To determine the cell size and DNA content, cells were fixed in 75% ethanol at 4°C, washed with PBS, and incubated at 37°C for 20 min in PBS containing 1% FBS, 0.1% Triton X-100, and 250 μg/ml RNAase A before staining with propidium iodide for FACS analysis. Cell viability of H_2O_2-treated cells was quantitated by trypan blue exclusion. Etoposide-mediated apoptosis was determined by propidium iodide staining for apoptotic cells for the sub-G1-DNA content and quantitated by FACS analysis.

Supplemental Data

Supplemental Data include experimental procedures and three figures and can be found with this article online at http://www.cell.com/cgi/content/full/127/1/125/DC1/.

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